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A Soluble Recombinant Multimeric Anti-Rh(D) Single-Chain Fv/CR1 Molecule Restores the Immune Complex Binding Ability of CR1-Deficient Erythrocytes

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CR1 (CD35, the C3b/C4b receptor) is a widely distributed membrane glycoprotein with a unique cluster conformation on the surface of erythrocytes (E). CR1 on E is responsible for the transport of immune complexes (IC) to liver and spleen. As a cofactor of the C3b cleavage by factor I, CR1 is also a potent inhibitor of C activation and inflammation. In some diseases (systemic lupus erythematosus, hemolytic anemia, AIDS, etc.) an acquired low level of CR1 on E has been observed, leading to an impaired clearance of IC. The aim of this study was to design a heterofunctional molecule that will bind to E and restore a normal or a supranormal CR1 density on E that could mimic the unique distribution pattern of CR1 on normal E. For that purpose a new multimerizing system based on the properties of the C-terminal part of the α-chain of the C4 binding protein (C4bp) was used. We first produced a multimeric soluble CR1 that proved to be a better inhibitor of in vitro C activation than the monomeric form of CR1, then a heteromultimeric molecule made of CR1 and single-chain Fv anti-Rh(D) valences able to attach E and providing E with as much as a 10-fold increase in CR1 density with the same CR1 distribution pattern as native E. CR1/single-chain Fv anti-Rh(D)-treated E were able in vitro to attach as many opsonized IC as native E. These data open the way for future use of multimeric and heteromultimeric forms of soluble recombinant CR1 as therapy of IC diseases. *The Journal of Immunology,* **2000, 164: 1505–1513.**

The receptor for C3b/C4b (CR1, CD35) is a widely distributed membrane glycoprotein present on polymorphonuclear leukocytes, macrophages, B lymphocytes, some T lymphocytes, follicular dendritic cells in lymph node germinal centers, glomerular podocytes, and erythrocytes (E) [3] (1, 2).

In primates, including man, CR1 on E (CR1/E) transports C3b opsonized IC from the circulation to liver and spleen (3). Immune adherence of IC to E depends on the number of molecules of CR1/E (4). CR1 density on E is controlled by a codominant autosomal gene related to a *Hin*dIII polymorphism located in an intron of the CR1 gene (5). CR1 is expressed on E at lower density (200–1000 sites) compared with leukocytes (20,000 sites). However, the organization in clusters of CR1 on E membrane (see the 1988 "cover of the year" of the *Complement and Inflammation Journal* (6)) favors high avidity multimeric ligand-receptor interactions. Thus, despite the low number of CR1 molecules expressed on each E, CR1 enables E to be the main agent of IC clearance. CR1 also serves as a cofactor of factor I for C3b and C4b cleavage and inactivation (7). Physiologically CR1 density decreases during aging on normal E (8). E from patients suffering from systemic lupus erythematosus (SLE) (9, 10), some hemolytic anemia (11), and HIV infection share an acquired reduction of the expression of CR1 (12–14). This low density of CR1 on E contributes to impairing IC transport mechanisms in patients with SLE, resulting in deposition of IC outside liver or spleen, which may induce tissue damage in susceptible organs, particularly kidney (15, 16).

In addition, inappropriate addressing of Ag-containing IC leads to an impaired Ab response to the Ag entrapped in IC. Complement consumption on vasculitis induced by IC precipitation leads to a down-spiraling process regarding clearance of IC; IC are poorly opsonized due to C deficiency, poorly transported due to CR1 deficiency on E, and only induce a poor Ab response to the Ag contained in IC, unable to clear them from the circulation.

Restoring the number of CR1/E may be an important new way for therapy of human IC-mediated diseases (14). In a monkey model, the CR1 density on E has been enhanced by bleeding these animals to increase the proportion of young E (17). However, possibly due to the practical limitation of this model, no significant effect on experimental IC nephritis was observed. Another system has been proposed to enhance the IC capture ability of E using bispecific Abs, one valence being directed against CR1, and the other against the Ag of interest. In experimental models in monkeys, capture of bacteriophage ϕX174 containing complexes has been demonstrated (18, 19). In addition to its technical complexity this model suffers from two major limitations: 1) bispecific Abs cannot be used when CR1 density is already decreased on E; and

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[3] Abbreviations used in this paper: E, erythrocyte; anti-Rh(D), anti-rhesus(D); DHFR, dihydrofolate reductase; AE, activated erythrocyte; C4bp, C4 binding protein; IC, immune complexes; multiCR1, multimeric CR1; multiRH1, multimeric scFv anti-Rh(D); NHS, normal human serum; scFv, single chain Fv; sCR1, soluble CR1; SCR, short consensus repeat; SLE, systemic lupus erythematosus; VBS, veronal-buffered saline; MFC, mean fluorescence channel.

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2) the precise Ag to be cleared must be known, and the corresponding specificity should be introduced into the bispecific Ab construction.

A more direct approach to restore the IC transportation capacity of E would be to attach recombinant CR1 directly on E. A monomeric soluble CR1 recombinant molecule has been produced (sCR1) (20). In vitro, sCR1 is a powerful inhibitor of human complement activation. *In vivo*, sCR1 is able to reduce the consequences of inflammation in some experimental models, such as postischemic myocardial inflammation, and in organ transplantation (21–23).Unfortunately, due to its weak affinity for C3b and short in vivo half-life, sCR1 has not demonstrated a beneficial effect in IC-mediated diseases.

Direct chemical linkage of monomeric CR1 randomly cross-links anywhere throughout the CR1 molecule to other E surface molecules and is unlikely to reproduce the tridimensional cluster long chain structure of CR1 on E demonstrated by the typical patchy staining of E for CR1 using anti-CR1 mAbs. Requirements for realistic future immunointervention restoring a physiological or a supraphysiologic density of functional CR1 on E are to obtain multimeric clusters of CR1 and to attach them easily on E by using an Ag-Ab ligand receptor interaction. The design of a recombinant bispecific anti-E/CR1 molecule was undertaken for that purpose.

Because existing multimerizing systems have not been found satisfactory, a new multimerizing system for recombinant protein has been developed to fulfil the required criteria for future in vivo use of soluble bifunctional multimeric molecules.

The multimerizing system should originate from a normal plasma molecule to avoid immunization, should lack any biological function, particularly complement activation or cell surface receptor interactions, and should confer at least five valences or more.

Despite their ability to induce multimerization, previous systems based on Fcγ (24), Fcμ (25), leucine zipper (26), chemical polyethylene glycol linkage (27), diabodies (28), streptavidin (29), and protein A (30) failed to pass these criteria. Some multimerizing proteins such as Fc or diabodies are direct activators of the complement cascade. Leucine zipper-based proteins are likely to be immunogenic and poorly soluble as originating from an intracellular protein as well as poorly stable and difficult to purify since they are noncovalently linked.

A new system fulfilled the criteria for a fair multimerizing system. The C4bp molecule is a spider-like structure made of seven α-chains and one β-chain (31). The basic repetitive structure of both chains is termed short consensus repeat (SCR). Each SCR of about 60 aa includes two intrachain disulfide bridges. Binding sites for C4b are located on the four N-terminal SCR of the α-chains, whereas the S-protein binding site is located on the two N-terminal SCR of the β-chain (32). Minor forms made from only seven α-chains or five α/1 β-chain molecules have also been described and found at a lower concentration in normal human plasma (32, 33).

The C-terminal part of the complement binding protein C4bp lacks biological function, but is responsible for the polymerization of the molecule in the cytoplasm of C4bp-producing cells (32). Due to this property and the lack of biological functions as well as the probable nonimmunogenicity of a normal human plasma protein, the C-terminal part of the α-chain of C4bp was chosen to set up homomultimeric and heteromultimeric proteins.

cDNA containing the C-terminal part of the C4bp coding sequence can be fused in 3′ with the sequence coding for the protein of interest, then transfected in cells as a single-phase construct. The chimera protein spontaneously multimerizes in the cytoplasm of transfected cells that secrete covalently linked multimeres (34). Cotransfection of cells by two different vectors containing sequences coding for two distinct multimeric molecules was believed to lead to the production of a heteromultimeric protein with valences from both molecules covalently linked together by disulfide bridges.

A C4bp C-terminal fragment-based multimeric soluble CR1 recombinant molecule was initially developed, then an heteromultimeric molecule composed of anti-rhesus(D) (anti-Rh(D)) scFv and CR1 valences was prepared and tested for fixation on E and ability to restore or to enhance IC capture by treated E.

Materials and Methods

Cloning of human anti-Rh(D) heavy and light chain variable region coding sequences and assembly into an scFv

A lymphoblastoid cell line, H2D5D2F5 (referred to as DF5), derived from the peripheral blood lymphocytes of a hyperimmunized donor (35) and producing a human monoclonal IgG1λ, specific for erythrocyte Rh(D) Ag, was used as source of monoclonal cells to rescue V_H and V_L regions via PCR amplification.

The single-chain Fv (scFv) was constructed according to the method described by Marks et al. (36). Briefly, total RNA was extracted from the lymphoblastoid cells, cDNA was obtained using the first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden) and pd(N)6 random primers. Amplification of V_H and V_L coding fragments was effected with the specific V gene family primers described by Marks et al. (37): GTG CAG HuVH1aBACK (5′-CAG GTG CAG CTG TCT GG-3′) and HuJH4–5FOR (5′-ACC TAA AAC GGT GAG CTG GGT CCC-3′) for the heavy chain; and HuVλ1BACK (5′-CAG TCT GTG TTG ACG CAG CCG CC-3′) and HuJλ2–3FOR (5′-ACC TAG GAC GGT CAG CTT GGT CCC-3′) for the light chain.

PCR assembly of the paired V_H and V_L with a $(Gly^4\text{-}Ser)^3$ linker and *Sfi*I and *Not*I restriction site addition was performed as described by Marks et al. (36, 37). The assembled structures were cloned into pHEN1 (38), (vector provided by G. Winter, Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.). The ligation product was introduced into *Escherichia coli* HB2151 by electroporation, and insert-containing clones were tested for the expression of soluble scFv following isopropyl β-D-thiogalactoside (IPTG) induction (36). The scFv construct from a clone with high specific agglutinating reactivity to anti-Rh(D) erythrocytes was selected for construction of the chimeric protein.

Primers used to amplify the C-terminal part of the C4bp molecule

A 169, or 202 when including an Myc tag, base pair C-terminal C4bp α fragment for scFv anti-Rh(D)/C4bp was amplified using the following primers, including, or not, a segment coding for a Myc tag inserted between anti-Rh(D) scFv and C4bp fragments: 5′ primer with Myc tag, 5′-AGTGCGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCT GAATGAGACCCCCGAAGGCTGTGA-3′; 5′ primer without Myc tag, 5′-AGTGCGGCCGCAGAGACCCCCGAAGGCTGTGA-3′; and 3′ primer, 5′-CTCGCGGCCGCCTCGAGTTATAGTTCTTTATCCAAAGTGG-3′. Underlined sequences represent restriction endonuclease sites. The 5′ and 3′ primers contain *Not*I and *Xho*I sites, respectively. The sequence depicted in bold characters codes for a EQKLISEEDLN Myc tag peptide. The amplified sequence contained at its 3′ end a stop codon and a restriction site for *Xho*I as well as at its 5′ end a restriction site for *Not*I.

A 167-bp C-terminal C4bp α fragment for CR1/C4bp was amplified using the following primers: 5′ primer, 5′-CCGAGACCCCCGAAGGCT-GTGA-3′; and 3′ primer, 5′-CTCGCGGCCGCCTCGAGTTATAGT-TCTTTATCCAAAGTGG-3′. The underlined sequence represents restriction endonuclease site. The 3′ primer contained a *Not*I site.

PCR amplification

Genomic DNA was used as a template for PCR amplification. A 100-μl reaction mixture was prepared, containing 500 ng of DNA, 500 ng of 5′ and 3′ primers, 16 nM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 2 U of AmpliTaq DNA polymerase (Perkin-Elmer, Roissy, France), then subjected to 30 cycles of amplification using a GenAmp PCR System 9600 (Perkin-Elmer). Cycles were 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. PCR products were analyzed by electrophoresis on a 2% agarose gel. Following that control, DNA was extracted

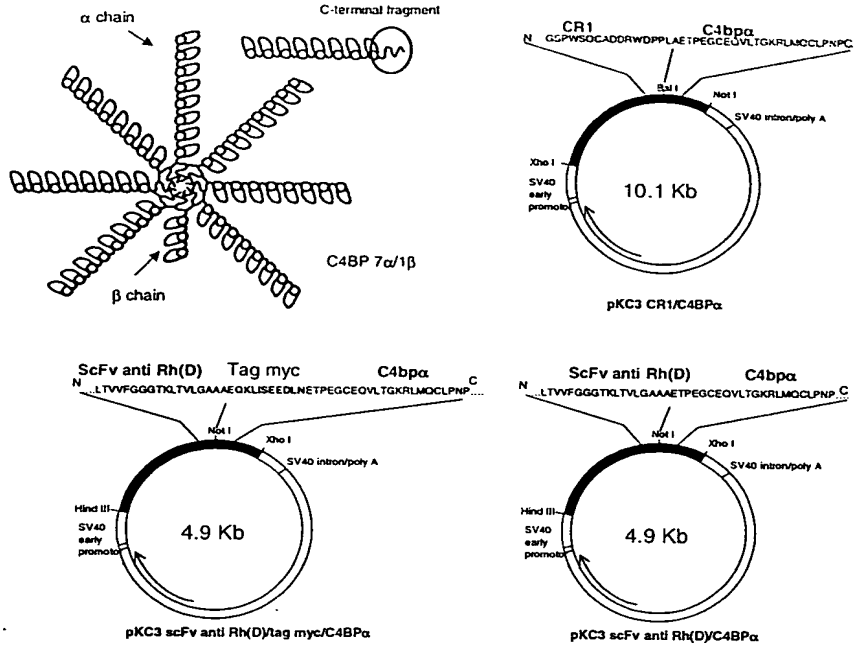

FIGURE 1. Maps of pKC3 CR1/C4bpα and pKC3 scFv anti-Rh(D)/C4bpα plasmids coding for multiCR1 and multiRH1, respectively. Amino acid sequences of the junction areas between scFv anti-Rh(D), CR1, and the C-terminal part of C4bp α-chain are depicted *above* the overall plasmid.

once with phenol/chloroform and once with chloroform, ethanol precipitated, and resuspended in 100 μl of water before digestion with *Not*I and *Xho*I for scFv anti-Rh(D) or with *Not*I for CR1.

Cloning of the CR1/C4bp (multiCR1) and anti-Rh(D) scFv/C4bp (multiRH1) constructs

Restriction enzymes and alkaline phosphatase used for cloning were purchased from Roche (Meylan, France). The T4 DNA ligation kit used was from Ozyme (Montigny-Le-Bretonneux, France). CR1 cDNA coding for the soluble extramembraneous part of CR1 (given by W. Wong Harvard Medical School, Boston, MA) was inserted in pCDM8 vector (Invitrogen, Leek, The Netherlands). The cDNA coding for CR1 was digested by *Xho*I and *Bal*I. The matching amplified C4bp fragment was digested by *Not*I, leading to 5′ blunt and 3′ cohesive end ligations. The DNA construct coding for the anti-Rh(D) scFv was digested by *Hin*dIII and *Not*I. The matching amplified C4bp fragment was digested by *Not*I and *Xho*I. For each construct, two DNA fragments were linked in a dephosphorylated pKC3 vector (Fig. 1).

Transfections of CHO eukaryotic cell line and amplification of the transfected genes

Transfection of pKC3 CR1/C4bpα construct was performed using a calcium phosphate transfection kit (5 Prime-3 Prime, Tebu, Le Perray en Yvelines, France), then selected for multiCR1 expression by limiting dilution and screening for CR1 secretion by ELISA. Plasmid pKC3 scFv anti-Rh(D)/C4bp was cotransfected with the dihydrofolate reductase (DHFR)-selective plasmid ST4 in a multiCR1 secreting clone. After limiting dilution multiCR1/scFv anti-Rh(D)-secreting clones were screened by flow cytometry for their ability to bind papain-treated E and to attach CR1 onto them.

Cell cultures

CHO DHFR-deficient (DHFR−; ATCC CRL-9096, American Type Culture Collection, Manassas, VA) were cultured in Ham's F-12 (Life Technologies, Cergy Pontoise, France) supplemented with glutamine (2 mM), 10% FCS (Life Technologies), penicillin/streptomycin/fungizone (1000 U/ml; 1000 μg/ml; 2.5 μg/ml). CHO DHFR− transfected by pKC3 CR1/C4bpα and sT4-DHFR were cultured in Ham's medium without thymidine and hypoxanthine (Biochrome, l'Aigle, France) supplemented with 10% heat-inactivated dialyzed FCS (Life Technologies), penicillin/streptomycin/fungizone (1000 U/ml; 1000 μg/ml; 2.5 μg/ml), and glutamine (2 mM).Cells transfected by pKC3 CR1/C4bpα, pKC3 scFv anti-Rh(D)/C4bpα, sT4-DHFR, and pMAMNeo were cultured in Ham's medium without thymidine and hypoxanthine supplemented with 10% heat-inactivated dialyzed FCS, penicillin/streptomycin/fungizone (1000 U/ml; 1000 μg/ml; 2.5 μg/ml), glutamine (2 mM), and G418 (700 μg/ml; Sigma, St. Louis, MO).

Antibodies

mAb anti-CR1 (J3D3 and J3B11) and mAb against Myc Tag (9E10, ATCC CRL-1729) were purified from mouse ascites using the octanoic acid contraprecipitation method (39). Hybridoma cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FCS, glutamine (2 mM; Life Technologies), penicillin/streptomycin/fungizone (1000 U/ml; 1000 μg/ml; 2.5 μg/ml; Sigma). J3D3 and J3B11 cells lines were a gift from E. Fischer (Institut National de la Santé et Recherche Medicale, Unit 430, Paris, France). Rabbit anti-human E polyclonal Abs were purchased from Cappel Laboratories (Cochranville, PA). Rabbit anti-BSA polyclonal Abs were purchased from Sigma. Biotinylated goat anti-streptavidin Abs were purchased from Biosys (Compiegne, France).

Biosynthetic cell labeling and immunoprecipitation

Cells were cultured for 1 night, in RPMI 1640 without cysteine and methionine (ICN, Costa Mesa, CA) supplemented with 10% heat-inactivated FCS, glutamine (2 mM), penicillin/streptomycin/fungizone (1000 U/ml; 1000 μg/ml; 2.5 μg/ml), and 50 μCi of [^{35}S]methionine cysteine (Amersham, Orsay, France). Fifty microliters of goat anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway) were washed three times in PBS (Biomérieux, Marcy l'Étoile, France), then incubated with 15 μg of J3D3 mAb for 1 night at 4°C. Beads were washed three times in PBS. Transfected cell culture supernatants were incubated with beads for 1 night at 4°C. Washed beads were then resuspended in SDS-PAGE sample buffer for electrophoresis. Reduced and unreduced immunoprecipitates were subjected to electrophoresis in a 3% SDS agarose or acrylamide gel.

Quantitation of soluble CR1 by ELISA (CR1-ELISA)

J3D3 and J3B11 anti-CR1 mAbs recognizing different epitopes of the molecule were used to quantify soluble CR1 antigenic sites in CR1 ELISA. One hundred microliters of J3B11 mAb (1.5 μg/well in PBS, pH 7.9) was coated on polystyrene microwell plates (Nunc Maxisorp immunoplates, PolyLabo/Paul Block, Strasbourg, France) overnight at 4°C. Wells were washed three times in PBS containing 0.05% Tween-20, then saturated for 1 h at 37°C using 200 μl of PBS containing 2% BSA (Sigma) and 0.05% Tween 20. The samples containing CR1 to be quantified were diluted in PBS/0.05% Tween 20 and incubated for 2 h at 37°C. Wells were washed three times in PBS/0.05% Tween 20, then incubated with 100 μl of biotinylated J3D3 (0.05 μg/well) for 1 h at 37°C. Wells were washed again three times in PBS/0.05% BSA, then incubated with streptavidin (0.05 μg/well; Sigma) for 30 min at 37°C. After three washes, wells were incubated with 0.05 μg/well of biotinylated phophatase alkaline (Sigma) for 30

min at 37°C. After three washes, 150 μl of 4 mg/ml paranitrophenylphosphate substrate diluted in alkaline buffer (Sigma) was incubated for 30 min at 37°C. Absorbance was measured at 405 nm in each well on a microplate reader (SLT reader 340 ATCC, Salzburg, Austria); sCR1 was used as standard (a gift from E. Fischer).

Size determination of mCR1 by gel filtration

Sephacryl S-400HR (Pharmacia) was packed in a 1.6 × 70-cm column. The gel was equilibrated in 150 mM phosphate buffer, pH 7.4, supplemented with 1 M NaCl. Chromatography was performed at 4°C at a stable flow rate of 0.113 ml/min. One milliliter of culture supernatant was injected. Fractions were collected every 9 min and analyzed by ELISA for CR1 after their collection. Calibration curve of the column was established using aldolase (158,000 Da), catalase (232,000 Da), ferritin (440,000 Da), and thyroglobulin (669,000 Da). The predicted apparent m.w. for totally excluded molecules (when the elution volume was equal to the dead volume) was in accordance with manufacturer's gel specifications.

CR1 inhibition of complement-dependant lysis of E

Human E were sensitized by incubation with a rabbit polyclonal Ab directed against human E for 30 min at 37°C. One hundred million AE were incubated for 30 min at 37°C with 100 μl of C5-deficient human serum at a 300-fold dilution in VBS containing 0.15 mM Ca^{2+}, 0.5 mM Mg^{2+}, and various concentrations of sCR1 or multiCR1. After two washes in VBS containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}, AE were incubated with 100 μl of EGTA-normal human serum (NHS; diluted 1/10) for 10 min each time at 37°C. After centrifugation for 5 min at 700 × g, the 405-nm absorbance of the supernatant was determined using a microplate reader (SLT Reader 340; ATCC, Salzburg, Austria).

Molecular interaction analysis

Molecular interactions were analyzed using a surface plasmon resonance device (Fisons, Cambridge, U.K.). One hundred micrograms of goat anti-mouse Abs were immobilized on aminosilane cuvettes using an ethyl dimebhylaminopropyl carbodiimide/*N*-hydroxysuccinimide coupling kit (Fisons). Residual NHS groups were blocked with 1 M ethanolamine, pH 8.5, for 2 min. The cuvette was then washed in PBS buffer. One hundred micrograms of 9E10 mAb was added. After 4 min the coated surface was washed four times with PBS. Crude culture supernatant of multiCR1/scFv anti-Rh(D)-secreting cells was added. After 3 min the solid phase was washed four times in PBS. Finally, 20 ng of J3D3 mAb was added for 3 min. The solid phase was then washed three times in PBS. After washing, changes in refractive indexes, expressed as arcs per second, were correlated with the amount of protein bound to the cuvette following each incubation step.

Papain treatment of E

Papain used in this assay was obtained from Merck (Nogent-sur-Marne, France). One hundred microliters of Rh(D)-positive E was washed twice in PBS. Thirty-five microliters of a 0.1% papain solution was added to the pellet. After a 7-min incubation at 37°C, E were washed twice and resuspended in 4 ml of PBS.

Preparation of CR1 reconstituted E

Twenty microliters of a 2.5% solution of papain-treated E was incubated for 30 min at 37°C with 50 μl of pure or diluted culture supernatants of cells cotransfected by pKC3 CR1/C4bpα and pKC3 scFv anti-Rh(D)/C4bpα.

Immunogold labeling of calcium ionophore-treated E using J3D3 anti-CR1 mAb

Two microliters of calcium ionophore-treated pellet containing E was washed twice in ice-cold PBS including 1% BSA (5 min, 780 × *g*). E were incubated in 50 μl of PBS/1% BSA containing 2 μg of J3D3 mouse anti-CR1 mAb for 45 min at 4°C. E were washed twice in ice-cold PBS-1% BSA, then incubated for 45 min at 4°C in 50 μl of PBS/1% BSA containing 10 nm gold-labeled goat anti-mouse Ab (British Biocell International, Cardiff, U.K.; and Tebu; 10-fold diluted). E were then washed twice in ice-cold 1% BSA/PBS. As controls, treated E and untreated E were incubated by omitting J3D3 mouse anti-CR1 mAb.

Electron microscopy

J3D3 mouse anti-CR1 mAb and 10 nm gold-labeled goat anti-mouse Ab-immunolabeled E were fixed in PBS containing 1% glutaraldehyde and 2% formaldehyde for 2 h at 4°C, then washed twice in ice-cold PBS. E were postfixed in PBS including 1% OsO_4 (Merck, Rahway, NJ), then washed twice in ice-cold PBS. E were pre-embedded in 20 μl of PBS containing 5.6% BSA and 6.2% glutaraldehyde. Resuspension of pellets was carefully avoided. Pre-embedded pellets were collected; dehydrated in 70, 80, 90, and 95°C and absolute ethanol successively; then embedded in Epon (Merck) at 60°C for 48 h. Ultrathin sections (60–80 nm) were collected over copper grids. Grids were osmium tetraoxide counterstained and examined in a JEM 100C JEOL electron microscope (JEOL, Tokyo, Japan).

Indirect immunofluorescence and flow cytometry

E were analyzed for CR1 antigenic site density using indirect immunofluorescence and flow cytometry as previously described (40). Two million washed E were incubated for 30 min at 4°C with 2 μg of biotinylated J3D3 in PBS containing 1% BSA and washed three times in the same buffer. One and a half micrograms of streptavidin-PE (Tebu) or streptavidin-Alexa 568 (Molecular Probes, Eugene, OR) in 50 μl of 1% BSA/PBS, 1 μg of biotinylated anti-streptavidin Ab (Biosys), and 1.5 μg of streptavidin-PE in 50 μL of 1% BSA/PBS was then sequentially added and incubated with the cells for 30 min at 4°C. Stained E were fixed using 0.37% formaldehyde (Merck) in the washing buffer.

The kinetics of the binding of multiCR1/scFv anti-Rh(D) on E were also analyzed after 2-, 5-, 10-, 20-, and 30-min incubation in 10% IC containing serum from an SLE patient in acute flare or 10% human AB serum from healthy individuals.

Flow cytometry of stained cells was performed on a FACStar^{Plus} apparatus (Becton Dickinson, Mountain View, CA). At least 10,000 events for each sample were collected. Mean fluorescence channel was used to quantify the staining of each sample.

CR1 distribution pattern using confocal microscopy

Labeled cells were examined by confocal laser scanning microscopy using a Bio-Rad (Hercules, CA) MRC-1024 equipped with an argon/krypton laser and fitted with the appropriate filter block for detection of Alexa 568 fluorescence. Images were taken using simultaneous dual channel scanning and were transformed into projection views by using sets of 30 consecutive single optical sections. Immunofluorescence and phase contrast images were merged. On black and white prints, immunofluorescence staining looks like dark spots.

BSA iodination

BSA was iodinated following the chloramine T method of Hunter and Greenwood (41). Briefly, 0.5 mCi of ^{125}I (Amersham) was added to 10 μg of BSA in 20 μl of phosphate buffer (0.05 M) and 15 μg of chloramine T (Sigma). The solution was then mixed by vortexing for 30 s. Twenty micrograms of metabisulfite (Merck) was added. After mixing for 30 s, [^{125}I]BSA was separated from free ^{125}I and reducing agent by gel filtration chromatography (Quick Spin columns Sephadex G-50; Roche, Indianapolis, IN).

IC binding assay

Iodinated BSA was incubated in RPMI for 30 min at 37°C with rabbit anti-BSA Ab. IC were formed at a 4-fold Ab excess relative to the equivalence point.

Complement fragment incorporation (opsonization) of IC was conducted in normal human serum. Eighty million erythrocytes were incubated with 250 μl of NHS and 25 μl of IC. After incubation at 37°C for different times, cells were washed three times in ice-cold RPMI. The radioactivity of the pellet was measured in a gamma counter (Packard 1900 TR, Meriden, PA). Papain-treated E were used as a negative control.

Results

Establishment and characterization of multiCR1-secreting cell lines

CHO eukaryotic cells were transfected by multiCR1-containing plasmid pKC3 using the calcium phosphate method (42), batch-cultured in selective culture medium, checked for CR1 expression using anti-CR1 immunofluorescence detection, then cloned by limiting dilution. CR1 expression in cloned cells transfected by multiCR1 pKC3 plasmid was checked by ELISA after limiting dilution cloning. Selected clones were then subjected to increasing levels of methotrexate up to 1.26 μg/ml to improve multiCR1 secretion by gene duplication. Analysis of multiCR1 secreted by selected clones, performed on agarose electrophoresis after immunoprecipitation, revealed that in nonreducing conditions, multiCR1

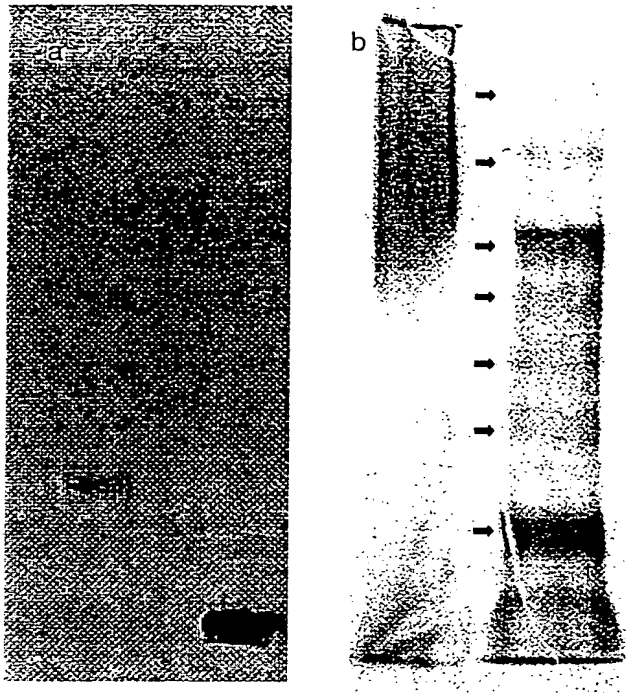

FIGURE 2. Analysis of multiCR1. Cells transfected by pKC3 CR1/C4bp were incubated overnight with [^{35}S]methionine and [^{35}S]cysteine as described in *Materials and Methods*. Culture supernatant was immunoprecipitated using J3D3 anti-CR1 mAb. Finally, immunoprecipitates were subjected to 4% SDS-PAGE under nonreducing (*left lane*) or reducing (*right lane*) conditions (*a*), and 3% agarose gel electrophoresis under nonreducing (*left lane*) or partially reducing (*right lane*) conditions (*b*). Proteins were visualized by autoradiography.

was secreted as a unique multimeric form, probably made of seven CR1 valences, as indicated by the seven-band pattern observed in partially reducing conditions (Fig. 2). Gel permeation analysis of culture supernatant containing multiCR1 was performed under nonreducing conditions showing a major homogenous peak of 1.650 kDa (Fig. 3). Degradation products with an apparent M_r lower than 50 kDa were also observed. The amount of degradation products was about 15% of the native multiCR1 peak.

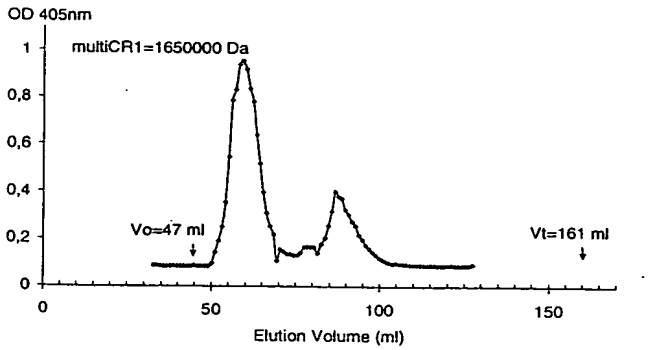

FIGURE 3. Gel permeation analysis of the apparent M_r of multiCR1 from culture supernatant of cells expressing multiCR1 (Vt, total volume; Vo, void volume). Apparent M_r was determined from the elution volume of multiCR1. CR1 concentrations were expressed as relative OD obtained by CR1 ELISA.

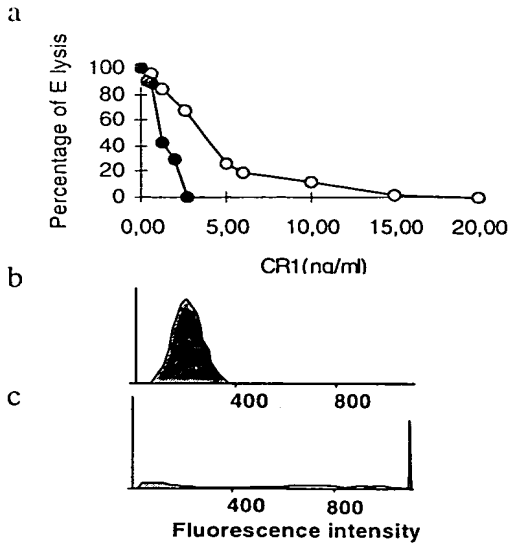

FIGURE 4. Inhibition of the C-dependent lysis of human activated EA by sCR1(○) and multiCR1 (●). EA were incubated 30 min at 37°C with C5-deficient human serum at a 300-fold dilution and with different concentrations of sCR1 and multiCR1. After two washings, EA were incubated with EGTA-NHS for 10 min at 37°C. E lysis was assessed by spectrophotometry at 405 nm. Flow cytometric analysis of multiCR1 binding on EA. *b*, Control staining omitting the opsonization step. *c*, Binding of multiCR1 on opsonized EA.

Analysis of complement inhibition

The abilities of multiCR1 and sCR1 to inhibit in vitro lysis of AE by complement were compared. A two-step complement activation system was used to obtain quantitative data comparing mono- and multimeric CR1 for their complement inhibition properties. E were sensitized using rabbit anti-E Ig and incubated with diluted C5-deficient serum to moderately opsonize E through C3b deposition in the presence of either monomeric or multimeric soluble CR1 as inhibitor. Finally, the final membrane attack pathway of complement was activated to obtain lysis of E, if opsonized. Both molecules showed an inhibitor activity, multiCR1 exhibited a higher inhibitory effect than sCR1, because lower concentrations were required for both 50% and total complement inhibition. MultiCR1 appeared to be a 5 times more potent complement inhibitor than sCR1 when considering a 50% inhibition level and a 10 times more potent complement inhibitor than sCR1 when considering total complement activation inhibition (Fig. 4*a*). MultiCR1 blocked complement activation exogenously, as established by the lack of complement inhibition as well as the lack of multiCR1 binding on E when multiCR1 was incubated with E then washed before the introduction of complement (Fig. 4*b*). Binding of multiCR1 on opsonized E was also verified using flow cytometry (Fig. 4*c*). This binding is in accordance with a mechanism of inhibition of hemolysis by multiCR1 through binding to C3b and exhibits a factor I cofactor activity.

Establishment and characterization of multiCR1/scFv anti-Rh(D)-secreting cell lines

Cells secreting multiCR1 were transfected by a C-terminal, C4bp-fused, scFv anti-Rh(D)-containing plasmid (Fig. 1). Clones expressing the multiCR1/scFv anti-Rh(D) chimeric molecule were selected after limiting dilution using flow cytometry and CR1 immunostaining on papain-treated E incubated with culture supernatants. Rh(D) Ag is known to be resistant to papain treatment as

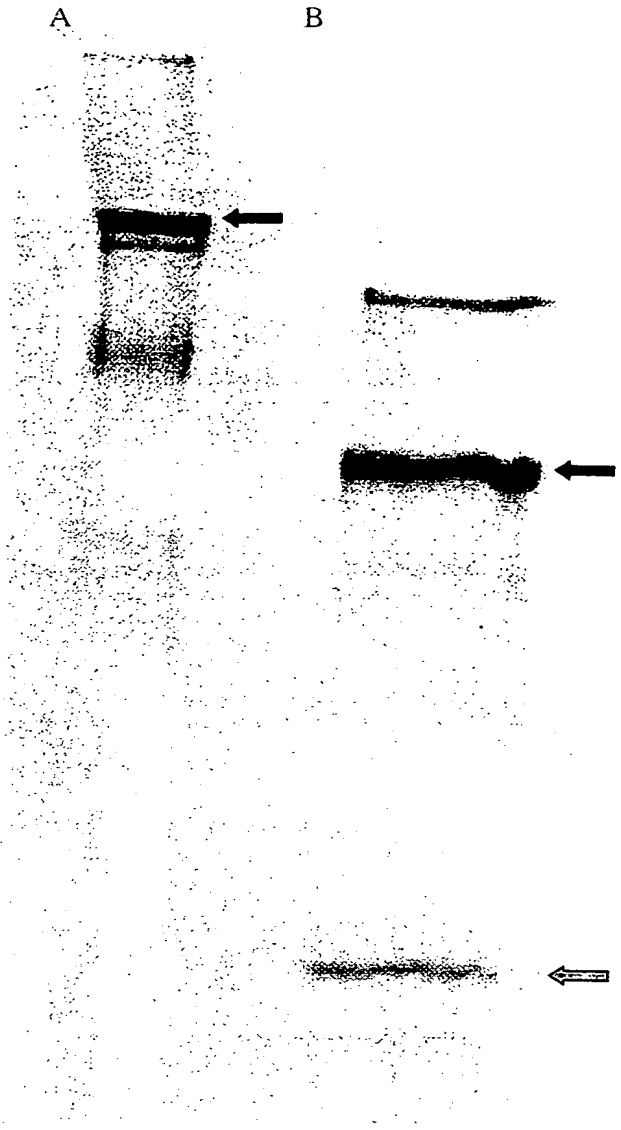

FIGURE 5. Analysis of multiCR1/scFv anti-Rh(D). Cells transfected with both pKC3 CR1/C4bpα and pKC3 scFv anti-Rh(D)/C4bpα were cultured overnight in the presence of [^{35}S]methionine-cysteine as described in *Materials and Methods*. Culture supernatants were immunoprecipitated using J3B11 anti-CR1 mAb. Immunoprecipitates were subjected to 6% SDS-PAGE under nonreducing (*A*) and reducing (*B*) conditions. Proteins were visualized by autoradiography. In *lane A*, a black arrow indicates the chimera molecule position. In *lane B*, black and gray arrows indicate CR1 and anti-Rh(D) scFv positions, respectively.

well as more accessible to Ab binding on papain-treated E. Analysis of multiCR1/scFv anti-Rh(D) chimeric molecule by SDS-PAGE, after immunoprecipitation using J3D3 anti-CR1 mAb, showed that this molecule is made from both scFv anti-Rh(D) and CR1 valences (Fig. 5). Surface plasmon resonance study using a solid phase coated with 9E10 anti-Myc tag mAb and sequential binding of the chimeric molecule followed by J3D3 anti-CR1 mAb also demonstrated the presence of both anti-Rh(D) and CR1 moieties in the same chimeric molecules (Fig. 6).

Microscopy analysis

Immunofluorescence staining for CR1 of treated or untreated E revealed a cluster distribution of multiCR1/scFv anti-Rh(D) at the

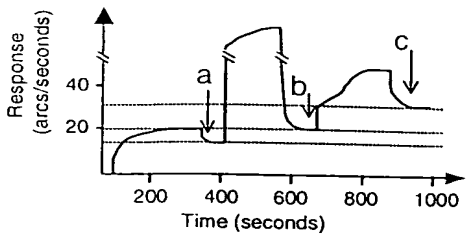

FIGURE 6. Analysis of molecular interactions using the Fisons IAsys apparatus. Sensorgrams showed residual fixations after washing of 9E10 anti-Myc tag mAb to an anti-mouse IgG Ab (*a*), then capture of multiCR1-scFv anti-Rh(D) by 9E10 anti-Myc tag mAb (*b*), and finally, the residual fixations after washing of J3D3 anti-CR1 mAb on the multiCR1-scFv anti-Rh(D) heteromultimeric molecule (*c*).

surface of treated E. Although the sizes of native and recombinant CR1 clusters were similar, the concentrations of CR1 cluster on treated E were greater than those observed on native E. Control immunofluorescence staining for Rh(D) Ag of papain-treated E (Fig. 7*a*) showed homogeneous staining, demonstrating that no

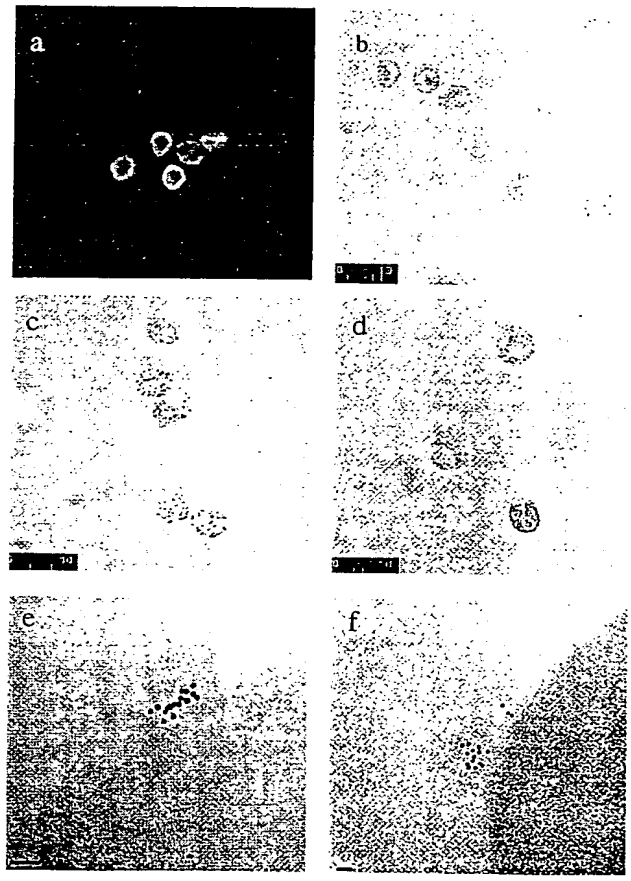

FIGURE 7. CR1 distribution pattern on E. *a*, As a control of the distribution of Rh(D), E have been labeled using DF5 anti-Rh(D) mAb. *b*, CR1 staining of E bearing 461 CR1/E. *c*, CR1 staining of E bearing 800 CR1/E. *d*, CR1 staining of CR1 depleted by papain treatment, then reconstituted using multiCR1/scFv anti-Rh(D). Control Rh(D) staining was photographed under UV epifluorescence illumination microscopy. CR1 stainings were obtained using confocal microscopic examination. Immunofluorescence staining appeared as dark spots. Electron microscopic examination of immunogold-stained E, using J3D3 anti-CR1 mAb, of native E (*e*), or of multiCR1/scFv anti-Rh(D) reconstituted E (*f*). Bar = 0.1 μm.

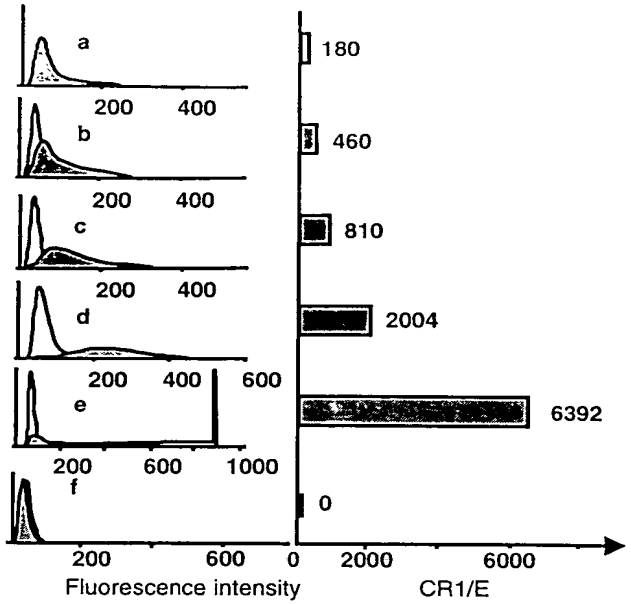

FIGURE 8. Enumeration of CR1 antigenic sites on E using flow cytometry analysis. E from three different normal individuals exhibiting low (*a*), medium (*b*), and high (*c*) CR1/E density were used to draw a standard curve. The mean number of CR1/E was deduced by interpolating mean fluorescence channel values observed from the reference curve. Low density native (nonpapain-treated E) cells were incubated with multiCR1/scFv anti-Rh(D) for 30 min at 37°C (*d*). CR1-depleted E were treated with papain for 7 min at 37°C (*f*), then incubated for 30 min at 37°C with multiCR1/scFv anti-Rh(D) (*e*).

cluster effect was related to Rh(D) distribution.

Transmission electron microscopy was used to further compare the cluster distribution of CR1 on treated or untreated E using CR1 immunostaining and gold-labeled second Ab revelation. The fixation of anti-CR1 mAb on CR1 clusters on papain-treated, then multiCR1/scFv anti-Rh(D)-coated, E led to the same gold particle-staining pattern as that observed in untreated native E (Fig. 7, *e* and *f*).

CR1 antigenic sites enumeration on native and multiCR1/scFv anti-Rh(D)-treated E using flow cytometry

Quantitative flow cytometry was used to assess CR1 antigenic sites on E. CR1 antigenic sites were quantified on native E exhibiting various CR1 densities, papain-treated E, and native or papain-treated E coated with multiCR1/scFv anti-Rh(D). On papain-treated E, CR1 was totally removed, while rhesus Ags were resistant to papain treatment and more accessible at the E surface. MultiCR1/scFv anti-Rh(D) not only enhanced CR1 density on native E of low CR1 density from 180-2000 CR1/E, but also induced largely supraphysiologic levels of CR1 on papain-treated E (Fig. 8).

In vitro binding of multiCR1/scFv anti-Rh(D) on E was identical in the presence of IC containing serum from an SLE patient in acute flare or in the presence of serum from normal healthy individuals, demonstrating that the binding of multiCR1/scFv anti-Rh(D) on E through scFv fixation is not impaired by IC binding in the liquid phase through the CR1 part of the molecule (data not shown).

IC binding study

Binding kinetics of opsonized ^{125}I-iodinated IC on 601 CR1/E, 800 CR1/E or papain-treated then multiCR1/scFv anti-Rh(D)-reconstituted E were analyzed. The ability of papain-treated, multiCR1/scFv anti-Rh(D)-coated E to bind IC was assessed using ^{125}I-labeled IC, prepared using [^{125}I]BSA and rabbit anti-BSA Abs. MultiCR1-reconstituted E bound IC better than native E, whereas E depleted of CR1 by papain treatment were no longer able to bind IC (Fig. 9).

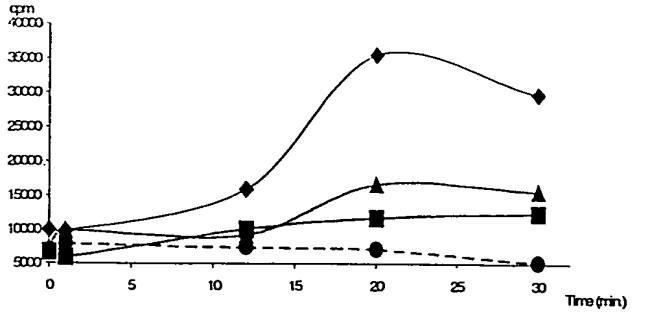

FIGURE 9. Binding kinetics of opsonized ^{125}I-iodinated IC on E. E with 460 CR1/E (■) or 800 CR1/E (▲), multiCR1/scFv anti-Rh(D) reconstituted E (◆), and papain-treated control E (●) were incubated for various periods of time with IC and NHS at 37°C.

Discussion

Primates, including humans, use CR1 on E as the main system for opsonized IC handling (3), inactivation, and transportation from blood to spleen and liver (43, 44). The role of CR1 on E is not only to prevent IC precipitation and vasculitis, but also to allow correct addressing of Ags contained in IC to optimize Ab response against a given Ag (45). In some pathological conditions an acquired decrease in CR1 on E is observed (9–12). In SLE, the decrease in CR1 on E is a part of a down-spiraling phenomenon of self-aggravating damaging processes. The decrease in CR1 on E impairs handling of opsonized IC as well as C3b inactivation and also allows complement consumption on precipitated IC, damaging vessels. In addition, less complement than in healthy individuals is already available in many SLE patients carrying one or several C4 null alleles representing SLE susceptibility genes (46). Ab responses that are weak among SLE patients are also impaired by poor opsonization and random addressing of Ags when IC precipitate, leading to the chronic presence of IC, a situation that in normal individuals is only transient (47). The mechanism of CR1 decrease on E in SLE patients is not totally clear. Proteolysis at the cell surface and exocytosis by vesicle formation have been proposed (48–51), although an abnormal regulation of CR1 density on E cannot be totally ruled out.

Nevertheless, restoring CR1 density on E might be an attractive way to interrupt the self-aggravating process of poor Ab response, complement consumption, and IC precipitation that leads to vasculitis and glomerulonephritis in SLE patients. Protein substitutive therapy must be directly linked to E, because sCR1, although able to bind C3b or C4b and help factor I for inactivation of C3b, cannot serve as an IC transport and addressing system or protect E from C3b deposition when complement activation is not fully inhibited. However, soluble CR1 is fairly active in various inflammation animal models, such as experimental myocardial infarction (20), acute respiratory distress, or xenogenic heart transplantation (22), despite the fact that rather high concentrations of sCR1 are needed and that the sCR1 half-life in vivo is quite short ($t_{1/2}$ = ~100 min) (52). Due to the shape of CR1, a very long single-chain multiple SCR molecule, direct chemical cross-linking of CR1 on E was likely to lead to a very poor yield of functional molecules. A method to attach CR1 on E in the same three-dimensional distribution pattern as native CR1 was sought. We considered a method

to produce multimeric CR1 and to attach these molecules to E that could prove a realistic means of future in vivo therapy. MultiCR1 fixation through an Ag-Ab reaction was chosen. We previously engineered an scFv anti-Rh(D) to make it into a multimeric recombinant molecule. We chose to use it to attach clusters of CR1 on E in the same multimeric molecule (34).

To multimerize CR1 or scFv anti-Rh(D), a minimal length α-chain C-terminal C4bp multimerizing fragment was used that fulfilled criteria for a multimerizing protein adapted for future in vivo use: originating from a normal human plasma protein, devoid of any biological function, secreted, soluble, covalently linked, and stable (34). In particular, unlike many other multimerizing systems, the C4bp-based system is not a complement activator and thus could be used not only to target cells for destruction but also for substitutive therapy, for imaging, or to provide anti-inflammatory molecules locally. A multimeric soluble recombinant CR1 molecule was first produced. The C-terminal part of the α-chain of the C4bp is efficient to induce polymerization during protein synthesis, and although the expression vector codes only for a monomer, multimers are assembled in the cell without the necessity of secondary modifications, resulting in the secretion of a unique covalently linked soluble molecule. After five rounds of methotrexate amplification, the multiCR1 concentration was around 2 μg/ml of culture supernatant, a commonly observed production yield in mammalian cells. The secretion of a very high M_r molecule (probably >1400 kDa) suggested that there is no size limitation using the C4bp-based multimerizing system. The perfect solubility and in vitro stability in culture supernatant of secreted multiCR1 were noteworthy, as also illustrated by a gel permeation curve (Fig. 3) showing a symmetrical peak without any retardation through protein/matrix interaction in the column. A longer lifespan of multiCR1 could also be expected, as it was a high M_r molecule, because albumin-receptor fused sCR1 has already demonstrated an in vivo half-life of 297 min (52), a 3-fold increase, compared with that of sCR1. The very high M_r of multiCR1 precluded an accurate size determination. In 4% PAGE-SDS, multiCR1 was detected as a single molecular species. Using agarose electrophoresis and partial reduction, a seven-band pattern was observed, suggesting a heptamer structure reminiscent of one of the physiological forms of C4bp in human plasma (53). An apparent M_r of 1650 kDa was obtained upon gel permeation chromatography that is also in accordance with the predicted M_r of a heptamer structure. We are presently unable to obtain definite structure imaging by electron microscopy due to the flexibility of the molecule and the various conformations of multiCR1. MultiCR1 retained its cofactor activity by enhancing C3b cleavage by factor I. In an Ig-sensitized E model of complement activation by Ig bound to E, the anti-inflammatory potency of multiCR1 as a complement activation inhibitor seemed to be better than that of sCR1, suggesting a better co-operative effect of C3b/C4b binding sites from different valences in multiCR1. The mechanism of complement inhibition by multiCR1 was further analyzed. Preincubation of EA with multiCR1 followed by washing did not lead to any deposition of multiCR1 on E, demonstrating again the lack of nonspecific interaction between multiCR1 and E (Fig. 4*b*). Flow cytometric analysis also showed the binding of multiCR1 on opsonized EA, illustrating the probable mechanism of complement inhibition by multiCR1 serving as a cofactor of factor I for C3b inactivation.

Considering that multimeric CR1 produced using the C-terminal C4bp multimerizing system was soluble homogenous and functional, we addressed in a second step the anchoring of multimeric CR1 on E using an Ag-Ab reaction. For that purpose, a heteromultimeric molecule made of CR1 and scFv anti-Rh(D) valences was designed and produced. This was performed by transfecting cells producing multiCR1 by a plasmid containing the scFv anti-Rh(D) C4bp α DNA construct. After cloning and limiting dilution, clones secreting heterofunctional multiCR1/scFv anti-Rh(D) were selected using immunofluorescent CR1 detection by flow cytometry on CR1-depleted, multiCR1/scFv anti-Rh(D)-reconstituted E. Heteromultimeric multiCR1/scFv anti-Rh(D) chimera retained the functional properties of both CR1 and scFv anti-Rh(D); they were able to bind E and to provide them with the ability to capture more opsonized IC than normal E used to do. Rh(D) Ag is an Ag of a relatively low density at E surface (5,000–10,000 Rh(D)/E), which is poorly accessible as made of 12 transmembrane spanning with short extracellular segments (54). However, for an experimental model we took opportunity of the dramatic increase in Rh(D) Ag presentation by papain treatment together with the removal of most other protein surface molecules, including CR1, to demonstrate that E totally depleted of CR1 can be reconstituted to physiological and even supraphysiologic CR1 density following incubation with heterofunctional multiCR1/scFv anti-Rh(D) molecules. It should be noted that the CR1 density reached through multiCR1/scFv anti-Rh(D) treatment is not dependant upon the natural CR1 density of a given E but, rather, upon the rhesus phenotype of that individual; the rhesus phenotype also leads to differences in Rh(D) density.

Electron microscopy and confocal analysis of immunostaining for CR1 clearly illustrated the cluster nature of the chimera molecule bound at the E surface, mimicking the natural conformation of CR1 at the E surface.

This heteromultimeric multiCR1/scFv anti-Rh(D) molecule was a preliminary model designed to explore the potential of the C4bp-based heteromultimeric molecules for therapy. It is clearly not a definite drug for human in vivo use. Moreover, even promising data from in vitro experiments can only be considered as a first step to the final goal of future in vivo therapy. In addition, Rh(D) Ag is not present in every individual and should be replaced during future development by a more common, more expressed, and more accessible Ag, such as the H Ag of the ABH system or another public E surface determinant expressed at a high level on E and restricted to these cells. Furthermore, the ratio of the two moieties of the molecule is not controlled in an α/α C4bp-based heterochimera. In the future, developments will include use of the C-terminal part of both α and β C4bp chains or use of a modified α-chain to control the ratio of two components in heterochimeric molecules and to modulate the polymerization process. Production system, vectors, leader sequences, and junction area at the 5′ end of the C4bp multimerizing fragment should also be optimized to meet the needs of high level production.

Nevertheless, the present research model established the feasibility of heteromultimeric molecules even of high M_r that remain soluble, functional, and able to bind specifically to an Ag-displaying cell surface. Improved anti-E/multiCR1 molecules could be used in the future for in vivo therapy in SLE or HIV-infected patients. Finally, this concept and the C4bp-based multimerizing system could be used in many domains when targeting of a specific molecule on a given cell type is needed, particularly when damages to the targeted cells are to be avoided.

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